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Title

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Permalink

<https://escholarship.org/uc/item/3941z0pm>

Journal

Leukemia, 29(3)

ISSN

0887-6924

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Publication Date

2015-03-01

DOI

10.1038/leu.2014.254

Peer reviewed



Published in final edited form as:

Leukemia. 2015 March ; 29(3): 615–624. doi:10.1038/leu.2014.254.

FHL2 regulates hematopoietic stem cell functions under stress conditions

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Abstract

FHL2, a member of the four and one half LIM domain protein family, is a critical transcriptional modulator. Here, we identify FHL2 as a critical regulator of hematopoietic stem cells (HSCs) that is essential for maintaining HSC self-renewal under regenerative stress. We find that *Fhl2* loss has limited effects on hematopoiesis under homeostatic conditions. In contrast, *Fhl2*-null chimeric mice reconstituted with *Fhl2*-null bone marrow cells developed abnormal hematopoiesis with significantly reduced numbers of HSCs, hematopoietic progenitor cells (HPCs), red blood cells and platelets as well as hemoglobin levels. In addition, HSCs displayed a significantly reduced self-renewal capacity and were skewed toward myeloid lineage differentiation. We find that *Fhl2* loss reduces both HSC quiescence and survival in response to regenerative stress, probably as a consequence of *Fhl2*-loss-mediated down-regulation of cyclin dependent kinase (CDK)-inhibitors, including *p21(Cip)* and *p27(Kip1)*. Interestingly, *FHL2* is regulated under control of a tissue specific promoter in hematopoietic cells and it is down-regulated by DNA hypermethylation in the leukemia cell line and primary leukemia cells. Furthermore, we find that down-regulation of *FHL2* frequently occurs in myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) patients, raising a possibility that *FHL2* down-regulation plays a role in the pathogenesis of myeloid malignancies.

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Online Supplementary Material: Supplementary Material and Methods and Data can be found with this article online.

Conflict of interest disclosure: The authors declare no competing financial interests

Introduction

Human *FHL2*, originally known as *DRAL*, was first identified as a four and a half LIM-domain protein that was down-regulated in rhabdomyosarcomas¹. The LIM domain, an acronym derived from *L*IN-11, *I*SL-1 and *M*EC-3, is characterized by a double zinc finger motif which mediates protein-protein interaction². *FHL2* belongs to the FHL protein family, a subfamily of LIM-only proteins, which includes the members FHL1, FHL3, FHL4 and ACT in humans³⁻⁶. *FHL2* is conserved between humans and mice with 91% amino acid identity. It is a potential adapter protein involved in multiple protein complexes⁶⁻⁸. *FHL2* interacts with a variety of transcription factors, including the androgen receptor, β -catenin, AP1, Smad, SKI, TRAF2, TRAF4, TRAF6, CREB, PLZF, Runx2, Foxk1, SRF, TUCAN and WT1, and functions as either a transcriptional coactivator or a corepressor in a cellular context-dependent manner⁹⁻²³, indicating that *FHL2* has important cellular functions.

FHL2 expression is often deregulated in cancer with down-regulation or overexpression in various types of tumors including rhabdomyosarcoma¹, prostate cancer²⁴, ovarian cancer²⁵, human melanoma¹³, lung cancer⁶, breast cancer²⁶ and liver cancer²⁷, suggesting that *FHL2* may act as an oncogene or as a tumor suppressor in a tissue-dependent manner. The dualistic nature of *FHL2* is also reflected by the fact that *FHL2* can act as a transcriptional repressor or activator depending on cell context². We previously found that *FHL2* is expressed in normal human CD34+ stem-enriched populations²⁸. Its function in hematopoiesis was first documented in our previous study examining overexpression of *FHL2* in bone marrow cells²⁸. However, the function of endogenous *FHL2* *in vivo* has not been reported yet.

To understand the biological role of *Fhl2* in hematopoiesis and HSC function *in vivo*, we characterized a *Fhl2* knockout mouse model. We found that *Fhl2* is essential for maintaining the function of HSCs by regulating the cell survival and quiescence of HSCs under regenerative stress, but that it has limited effects on hematopoiesis under homeostatic conditions. In addition, *Fhl2* loss leads to down-regulation of CDK inhibitors including p21(Cip1), p27(Kip1) and p57(Kip2) in HSC-enriched populations. However, we showed that forced expression of p21(Cip1) or p27(Kip1) but not p57(Kip2) in HSC-enriched population partially rescued *Fhl2* depletion-induced quiescence loss. We also found that *FHL2* is down-regulated in both MDS and AML patients, and have identified a tissue specific promoter of *FHL2* in hematopoietic cells. Of interest, our results revealed that down-regulation of *FHL2* is associated with DNA hypermethylation of *FHL2* hematopoietic specific promoter region, and it can be re-activated by hypomethylating agent in the KG1 myeloid leukemia cell line and primary AML cells. Together, these results suggest that *FHL2* is an important regulator of HSC self-renewal in response to stress, and that *FHL2* down-regulation is mediated by aberrant DNA methylation in a subset of AML patients, thereby contributing to leukemogenesis.

Materials and Methods

Mice and Blood Cell Counts

Fhl2^{-/-} knockout mice were bred into C57Bl/6 background for more than eight generations. All animal experiments were approved by the University of Illinois at Chicago Institutional

Animal Care and Use Committee. Peripheral blood (PB) samples were collected by tail bleeding into tubes containing EDTA. Complete blood counts and differentials were obtained using a Hemavet 950FS (Drew Scientific).

Flow cytometry

Bone marrow cells were obtained by flushing of femurs and tibiae in DPBS with 2% FBS. PB was obtained by tail bleeding. Phenotypic analyses of HPCs, LSK and HSCs have been described in our previous studies²⁹. The following mAbs were used: Streptavidin-PE-CY5, PE-Sca-1, APC-C-kit, PE-Cy7-CD48, APC-CD150, Streptavidin-APC-Cy7, APC-C-kit, PE-Cy7-CD16/32, e450-CD34, PE-CD45.1, FITC-CD45.2. A mixture of mAbs recognizing CD3e, B220, TER-119, CD19, Mac-1 and Gr-1 was used to identify Lin⁺ cells. All mAbs were obtained from eBioscience except CD150, which was from Biolegend. For lineage analysis, whole BM cells were stained with various combinations of antibodies for different cell populations: Percp-B220 and APC-IgM for B cells; PE-Gr-1 and APC-Mac-1 for myeloid cells; APC-Ter119 and PE-CD71 for erythroid cells; CD4 and CD8 for mature T cells; FACS analysis was performed using a CyAn ADP flow cytometer (Beckman Coulter). All data were analyzed by FlowJo software (TreeStar, Inc).

Cell cycle analysis and apoptosis

For Hoechst 33342/Pyronin Y staining, BM cells were stained with 1µg/mL Hoechst 33342 and 50µM Verapamil at 37°C for 45 mins, followed by staining with 1µg/mL Pyronin Y at 37°C for 45 mins. Subsequently, the cells were stained with mAbs against cell surface markers to identify the Lin⁻Sca-1⁺C-kit⁺ population. For DAPI staining, cells were stained with antibodies to identify HSCs, then fixed, stained with 5µg/mL DAPI. For the apoptosis analysis, freshly isolated BM cells were first stained with antibodies to identify Lin⁻Sca-1⁺C-kit⁺ cells, then washed in binding buffer and incubated with Annexin V APC and DAPI.

Homing assay for hematopoietic progenitor cells

In vivo homing assays were performed as described by Foudi et al. with some modifications³⁰. Briefly, 20×10⁶ BM cells from *Fhl2*^{-/-} or WT mice were suspended in 1 ml PBS with added CFSE to a final concentration of 2.5µM, incubate cells at 37°C for 15 minutes, then an equal volume of pre-warmed FBS (100%, filtered) was added to stop labeling. Cells were washed twice with PBS, followed by injection into the retro-orbital sinus of recipient mice that had been irradiated 24 hours before injection. Bone marrow was harvested 6 hours after injection, stained with antibodies against lineage markers and Sca-1, the frequency of CFSE⁺ cells in Lin⁻Sca-1⁺ population was determined by FACS. The formula is based on the percentage of CFSE⁺ Lin⁻Sca-1⁺ donor cells that homed to the BM as determined by flow cytometry (A), multiplied by the cellularity of recovered BM from the lethally irradiated recipients at 6 hours after transplantation (B), then divided by the number of CFSE⁺ Lin⁻Sca-1⁺ cells injected per mice (N): % homing= (A×B)/N.

Transplantation assays

For competitive repopulation assays, 2.5×10^6 BM cells (CD45.2) from *Fhl2*^{-/-} or *Fhl2*^{+/+} (control) mice were mixed 1:1 with the competitor BM cells (CD45.1/CD45.2) from B6.SJL/Ly5.1 mice and transplanted into lethally irradiated Ly5.1 mice by retroorbital injection. For generating BM chimeras, 10^6 BM cells from *Fhl2*^{-/-} or *Fhl2*^{+/+} (control) were injected into lethally irradiated Ly5.1 mice.

Quantitative real-time PCR

Total RNA was isolated from LSK cells of the transplanted *Fhl2*^{+/+} and *Fhl2*^{-/-} mice using an RNeasy Mini Kit (QIAGEN). And cDNAs were reverse-transcribed from total RNA prepared from LSK cells. Then cDNA was subjected to real-time PCR using SYBR Green Supermix (BIO-RAD) in realtime PCR System I-cycler (BIO-RAD). Amplification of β -actin was used for mice sample normalization. The housekeeping form of porphobilinogen dehydrogenase gene (HMBS)³¹ was used for human sample normalization. Primer sequences are listed in supplementary materials and methods.

5-Aza-2'-deoxycytidine treatment

KG-1 leukemia cells or primary leukemia cells were incubated for 72 hours in medium containing 2 μ M 5-aza-2'-deoxycytidine; the drug was replenished daily.

Bisulfite sequencing

Analysis of methylation of promoter region of bisulfite-treated KG-1 cells is described in supplementary materials and methods.

Retroviral production and infection

Retroviral constructs are described in supplementary material and methods. Retroviruses were produced as previously described²⁸. Bone marrow cells were collected from primary *Fhl2*^{+/+} and *Fhl2*^{-/-} transplantation mice (10 months after transplantation). The Lin⁺ cells from these BM cells were labeled with antibodies against cell surface markers, including Biotin-Mac-1, Gr-1, CD4, CD8, B220, and Ter119, and depleted with Streptavidin Dynabeads (Life Technologies). The resultant Lin⁻ population was infected with retrovirus as we previously described²⁸. The uninfected cells were killed after 2-day culture with 2.5 μ g/ml puromycin.

Patient Samples

The existed RNA and DNA samples from the MDS patients were obtained from Huashan hospital of Fudan University, Shanghai, China. The AML patient samples were obtained from the University of Illinois Hospital at Chicago. Informed consent was obtained in accordance with protocols approved by the review boards of participating hospitals.

Statistical analysis

Statistical significance was calculated using the two tailed Student's *t* test.

Results

Fhl2 loss has limited effects on hematopoiesis under homeostatic conditions

To determine the function of endogenous *Fhl2*, we conducted a loss of function study. Dr. Ju Chen (UCSD, CA) kindly provided *Fhl2*^{-/-} mice for this study (previously described³²). Briefly, the *Fhl2*^{-/-} mice were backcrossed for eight generations into the C57BL/6 background. The deletion of *Fhl2* allele was determined by PCR analysis of genomic DNA isolated from the *Fhl2*-deficient and control wildtype mice (Supplementary Figure S1A). *Fhl2*-null mice are viable and do not have obvious abnormal hematopoiesis. White blood cell (WBC) counts and hematocrits in cohorts of *Fhl2*-deficient mice and control wildtype littermates were monitored at regular intervals for signs of hematopoietic abnormalities. At 2 months of age, *Fhl2*^{-/-} mice revealed normal WBC, platelet, and red blood cell (RBC) counts and hemoglobin (Hb) levels in the peripheral blood (PB) as compared to control littermates. Within the WBC population, there were no differences in absolute lymphocytes, neutrophils, monocytes, basophils, or eosinophils (Supplementary Table S1) between *Fhl2*^{-/-} mice and control littermates. Analysis of the lineage distributions in bone marrow, spleen and thymus from *Fhl2*^{-/-} mice and control littermates by flow cytometric analysis revealed that *Fhl2*^{-/-} mice had a normal frequency of myeloid cells, B cells and T cells in these tissues (Supplementary Figure S1B & Figure S2). This data suggests that loss of *Fhl2* does not affect hematopoietic cell differentiation in young mice.

During normal hematopoiesis, the long-term HSCs (LT-HSCs) have a capacity to self-renew with the potential for differentiation to common myeloid progenitor (CMP) and more committed myeloid progenitor cells, including granulocyte-macrophage (GMP) and megakaryocyte-erythroid progenitor (MEP)³³. To determine whether *FHL2* is involved in the maintenance of HSCs/HPCs *in vivo*, we characterized the compartments of HSCs and HPCs by flow cytometric analysis. The frequency and total number of the stem cell enriched population LSK (Lin⁻Sca⁺c-Kit⁺) and LT-HSCs (Lin⁻Sca⁺c-Kit⁺CD48⁻CD150⁺) were slightly increased in *FHL2*^{-/-} mice as compared to wildtype mice (Figure 1A&B) whereas there was a statistically significant decrease in the number of HPCs in *FHL2*^{-/-} mice compared to *Fhl2*^{+/+} mice. To determine whether *Fhl2* loss affects the differentiation of HPCs, the subpopulations of myeloid progenitors in *Fhl2*^{-/-} and control mice were analyzed. As shown in Figure 1A&B, both *Fhl2*^{-/-} and *Fhl2*^{+/+} mice had comparable frequencies of CMP, GMP and MEP cells *in vivo*. However, the total number of GMP and MEP was slightly reduced in *Fhl2*^{-/-} mice as compared to control mice.

Given the fact that *Fhl2* regulates cell proliferation and apoptosis in various cell types³⁴⁻³⁷, we examined whether loss of endogenous *Fhl2* affects the proliferation and apoptosis of HSCs and HPCs *in vivo*. As shown in Figure 1C-1E, *Fhl2* loss slightly increases proliferation of HSCs but not LSKs and HPCs. However, it did not affect the apoptosis of HSCs and HPCs or the quiescence of HSCs (Supplementary Figure S3&4). Therefore, loss of *Fhl2* likely expands the HSCs by increasing the proliferation of HSCs and slightly inhibiting their differentiation into HPCs at steady state, as evidenced by a reduced number of HPCs and a slightly increased number of HSCs in BM cells.

Loss of *Fhl2* markedly reduces HSC self-renewal capacity after transplantation

Although loss of *Fhl2* affected the HSCs number and proliferation, but it did not significantly disrupt hematopoiesis in homeostatic state. This prompted us to test the role of *Fhl2* in the HSCs under stress. BM transplantation exposes HSCs to replicative, inflammatory and oxidative stresses^{38, 39}, eventually leading to loss of HSCs' capacity for self-renewal. We performed competitive repopulation assays, in which the same number of bone marrow (BM) cells (CD45.2) from *Fhl2*^{-/-} or *Fhl2*^{+/+} mice along with equal number of wild-type competitive BM cells (CD45.1CD45.2) were transplanted into lethally-irradiated syngeneic recipients (CD45.1). Thereafter, we performed serial transplantations in which BM cells mixed from 2-3 mice in each group were sequentially transplanted into serial lethally-irradiated syngeneic recipients (Figure 2A), thereby forcing to repeatedly self-renew. The frequency of donor-derived PB cells, which reflects the repopulation capacity of donor HSCs/HPCs, was analyzed every month after transplantation. The ratio of *Fhl2*^{-/-} derived (CD45.2+) vs. competitor-derived total PB cells (CD45.2+ /CD45.2+) was higher after primary transplantation than after secondary transplantation, and gradually decreased by 4-5-fold as compared to the ratio of *Fhl2*^{+/-}-derived vs. competitor-derived PB cells after tertiary transplantation (Figure 2B). Interestingly, 4 months after tertiary transplantation, we found the frequency of myeloid cells was increased at expense of B cells in *Fhl2*^{-/-} derived PB cells as compared to *Fhl2*^{+/-} derived PB cells. It is likely that *Fhl2*-null HSCs were skewed toward myeloid lineage differentiation (Figure 2C). Next, we determined the relative ratio of donor-derived vs. competitor-derived cells in Lin-, HPC, LSK, HSC in BM at 4 months after tertiary transplantation in both *Fhl2*^{-/-} and *Fhl2*^{+/-} chimeric mice. Consistent with these results, the *Fhl2*^{-/-} HSCs generated significantly lower cell numbers within all subsets of primitive hematopoietic cells than did *Fhl2*^{+/-} HSCs (Figure 2D). Collectively, these results indicate that *Fhl2*^{-/-} HSCs have a reduced self-renewal capacity after serial transplantation.

Loss of *Fhl2* leads to development of abnormal hematopoiesis in chimeric mice

As wildtype HSCs/HPCs may delay the development of functional defects of *Fhl2*-null HSCs *in vivo* in competitive assay, we generated *Fhl2*^{-/-} and *Fhl2*^{+/-} chimeric mice reconstituted with BM cells only from *Fhl2*^{-/-} or *Fhl2*^{+/-} mice. As determined by flow cytometric analysis 6 months post-transplantation (Figure 3A-B), more than 90-97% of BM cells or CD34-LSK HSCs from recipient mice were derived from donor BM cells, indicating a comparable engraftment ability of *Fhl2*^{-/-} and control BM cells in the recipient mice. We monitored these recipient mice by complete blood cell (CBC) analysis monthly. Interestingly, while both *Fhl2*-null and control chimeric mice displayed normal hematological parameters at 2 and 3 months post-transplantation (data not shown), the *Fhl2*-null mice started to develop abnormal hematopoiesis at 6 months post-transplantation, as evidenced by significantly decreased numbers of red blood cells and platelets as well as hemoglobin levels in *Fhl2*-null chimeric mice as compared to control chimeric mice (Figure 3C).

To determine whether the development of abnormal hematopoiesis in *Fhl2*-null chimeric mice results from reduced self-renewal capacity of *Fhl2*-null HSCs in response to regenerative stress, we characterized HSC and HPC compartments in chimeric mice at 7

moths post-transplantation. As shown in Figure 4, the HSCs, LSKs and HPCs were all decreased markedly in *Fhl2*-null chimeric mice as compared to control chimeric mice, suggesting that *Fhl2* loss impaired the repopulation capacity of HSCs *in vivo*. Consistent with our observation that the number of both red cells and platelets were decreased significantly in *Fhl2*-null chimeric mice as compared to control mice, the MEPs that eventually develop into megakaryocytes and erythroid cells were significantly reduced in the same mice. Thus, it is likely that *Fhl2* loss blocks the differentiation of megakaryocyte and erythroid cells at an early developmental stage under stress.

We also performed *in vivo* homing assays as we described previously²⁹. The *Fhl2* null and wildtype control BM cells had a comparable homing abilities, indicating that *Fhl2* loss does not affect the homing ability of BM cells (Supplementary Figure S5).

***Fhl2* is required for the maintenance of HSC quiescence under stress**

We observed an increase of proliferation of HSCs, but not HPCs, in young *Fhl2*-null mice. Consistently, the proliferation of HSCs and LSKs, but not HPCs, was increased significantly in *Fhl2*-null chimeric mice as compared to control mice (Figure 5A-5C). Given the fact that proliferation of HSCs is often associated with exhaustion of HSCs and loss of quiescence^{40,41,42}, we next examined the quiescence of HSCs in the chimeric mice. As expected, there were less *Fhl2*-deficient HSCs in G₀ phase than the control HSCs in chimeric mice (Figure 5D-5E). Moreover, the frequency of apoptosis was increased in *Fhl2*-deficient HSCs as compared to control HSCs in the same chimeric mice (Figure 5F). Therefore, it is likely that an increase in the proliferation of *Fhl2*-deficient HSCs, augmented by transplantation-induced stress, leads to loss of quiescence and reduced survival of *FHL2*-deficient HSCs, ultimately disrupting the function of *Fhl2*-deficient HSCs *in vivo*.

***Fhl2* loss leads to down-regulation of CDK inhibitors in HSC-enriched population**

To determine the molecular mechanism underlying the decreased quiescence in the *Fhl2*-null HSC-enriched population (LSKs), we performed qRT-PCR analysis of critical cell cycle regulators in the LSKs isolated from chimeric mice reconstituted with *Fhl2*^{-/-} and wildtype BM cells 6 months post-transplantation. As shown in Figure 6A, CDK inhibitors including p21(Cip), p27(Kip1) and p57(Kip2) were all down-regulated significantly in *Fhl2*-null LSKs as compared to wildtype LSKs, whereas *Cdk1*, *Cdk 2*, *Cdk3* and *Cdk6* were expressed at comparable levels in both *Fhl2*-null and control LSKs. Cyclin-dependent kinase (CDK) inhibitors include *p21 (Cip1)*, *p27 (Kip1)* and *p57 (Kip2)* are critical regulators of the quiescence and self-renewal of adult HSCs^{40, 43-45}. Interestingly, we found that forced expression of *p21 (Cip1)* or *p27 (Kip1)* but not *p57 (Kip2)* partially rescued the decreased quiescence of *Fhl2*-deficient LSKs (Figure 6B). These data suggest that down-regulation of CDK inhibitors, including *p21 (Cip1)* and *p27 (Kip1)*, contributes to the decreased quiescence of LSKs induced by *Fhl2* depletion.

Down-regulation of *FHL2* in a subset of AML and MDS patients

We found that *FHL2* was down-regulated in a subset of t-MDS/t-AML patients³¹, raising the possibility that *FHL2* may also be deregulated in a subset of *de novo* AML and MDS patients. We performed an analysis of the Affymetrix GeneChip dataset for 542 AML

patients and 74 healthy individuals published online (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE13159>) by Haferlach *et al*⁴⁶. The expression of *FHL2* is significantly downregulated in AML patients compared to the healthy individuals (Figure 7A). We also determined the expression of *FHL2* in BM cells from 46 unselected patients with MDS and 10 control individuals without evidence of MDS or hematological malignancies by qRT-PCR analysis. Overall, *FHL2* was down-regulated marginally in BM cells from patients with MDS compared to controls (Figure 7B). However, on average, there was about 4-fold down-regulation of *FHL2* in approximately 35% of *de novo* MDS patients as compared to controls. Together, these data indicate that downregulation of *FHL2* is detected in AML and MDS patients.

***FHL2* is regulated in a tissue-specific manner in hematopoietic cells**

To examine the expression of *FHL2* in CD34⁺ hematopoietic cells, we performed RT-PCR, and found that the full-length *FHL2* transcript is not expressed in CD34⁺ hematopoietic cells. However, these cells expressed an isoform of *FHL2* that does not contain the previously- recognized 5'exon (Figure 8A). 5'-RACE was performed to clone the 5' end of this isoform of *FHL2*, as well as the full-length transcript from bone marrow cDNA. The sequence data (GenBank ID: DQ307067) revealed that this isoform of *FHL2*, designated *B-FHL2*, encodes the same protein as *FHL2*, but has a different 5'-UTR. Comparison of *B-FHL2* to *FHL2* transcripts revealed that *FHL2* (gi: 42403584), *DRAL* (gi:11761688), and *B-FHL2* all encode the same peptide, but have unique 5' flanking sequences (Figure. 8A). A TATA box was identified -813bp upstream of the 5' sequences of *B-FHL2*, suggesting that the transcription of *B-FHL2* is initiated upstream of the 5' transcript sequences we cloned, and is regulated by a unique promoter in hematopoietic cells. To determine the tissue distribution of *B-FHL2* expression, we performed RT-PCR analysis of a multiple tissue cDNA panel, and found that *B-FHL2* is predominantly expressed in bone marrow and testis, is moderately expressed in placenta, and is not expressed in other tissues (Supplementary Figure S6).

Downregulation of *FHL2* in leukemia cells is associated with promoter hypermethylation

Alteration of DNA methylation is associated with aberrant gene silencing in cancer and leukemia⁴⁸. *FHL2* is under the control of a tissue-specific promoter in bone marrow (Figure 8A). A region containing a CpG island, was identified within the promoter region of *B-FHL2* (-1628bp to -1425bp upstream of the 5' end). qRT-PCR analysis of *FHL2* expression showed that *FHL2* was expressed at a markedly lower level in KG1 leukemia cells as compared to K562, HL60 and U937 leukemia cells (Supplementary Figure S7). Bisulfite sequencing analysis of the CpG island in the promoter region of *B-FHL2* revealed that 99.75% of the CpGs are methylated in untreated KG-1 cells, whereas 52.5% of CpGs are methylated in KG-1 cells treated with the demethylating agent, 5-aza-2'-deoxycytidine (5-aza-dC) (Figure 8B). Thus, this region is highly methylated in untreated KG-1 cells, and can be demethylated significantly after treatment with 5'-aza-dC. To determine if the reduced expression of *FHL2* in KG-1 cells is associated with the methylation status of the *FHL2* promoter, we performed real-time RT-PCR analysis of *FHL2* expression, and found that 5'-aza-dC-treated KG-1 cells have a 15-fold increase in expression of *FHL2* as compared to untreated KG-1 cells (Figure 8C). In addition, we also analyzed the *FHL2* expression in BM

cells from 11 AML patients and 2 healthy individuals. As shown in Figure 8D, *FHL2* expression was downregulated in BM cells from the majority of AML patients compared to healthy individuals. However, the promoter region of *FHL2* was hypermethylated in BM cells from 6 of 11 AML patients compared to healthy individuals, as determined by bisulfite sequencing (Figure 8E). The hypermethylation of *FHL2* promoter was correlated with downregulation of *FHL2* in BM cells from these six AML patients. To determine whether hypermethylation of *FHL2* promoter directly caused its downregulation, we compared the *FHL2* expression in the primary leukemia cells before and after treatment with 5'-aza-dC. We found that 5'-aza-dC markedly induced *FHL2* expression in leukemia cells with hypermethylation of *FHL2* promoter but not in the cells without *FHL2* hypermethylation (Figure 8F). Collectively, these data suggest that the reduced expression of *FHL2* in some AMLs is a result of hypermethylation of the promoter region.

Discussion

The role of endogenous *Fhl2* in hematopoiesis has not been reported. In this study, we identified a new role for *Fhl2* as a critical regulator of HSCs, essential for maintaining HSC quiescence and survival in response to regenerative stress.

Fhl2 has been implicated in tissue regeneration and repair. Deletion of *Fhl2* inhibits angiogenic functions of endothelial progenitor cells⁴⁹ while the lack of *Fhl2* perturbed skeletal muscle regeneration by down-regulating the myogenic progenitor cell activity⁵⁰. *Fhl2*-deficient mice displayed an impaired intestinal wound healing⁵¹ and skin wound healing⁵² as well as decreased activity of osteoblasts²¹. We have found that loss of *Fhl2* reduces the repopulation capacity of long-term HSC but not short-term HSC and progenitor cells, during hematopoietic regeneration. Competitive repopulation assays also confirmed that *Fhl2*-null HSCs have a reduced self-renewal capacity. Of note, this study reveals that *Fhl2* is involved in hematopoietic regeneration by directly regulating HSC self-renewal capacity. It will be interesting to determine whether *Fhl2* also regulates the activity of tissue-specific stem cells during regeneration of nonhematopoietic tissues.

The role of *Fhl2* in adult stem cells has not been previously reported. We found that *Fhl2*-null HSCs had a significantly reduced self-renewal capacity and had engraftment bias toward myeloid cells after serial transplantation, but *Fhl2*-null HSCs in primary young mice had limited functional defects in proliferation and differentiation, suggesting that *Fhl2* is critical for maintaining the self-renewal of HSC in response to regenerative stress but it is dispensable under homeostatic conditions. Quiescence and survival are critical for the maintenance of HSCs. Reduced quiescence of HSCs often results in HSC exhaustion⁵³. We find that *Fhl2* loss does not affect quiescence and survival of HSCs and HPCs in the homeostatic state, but it significantly reduces quiescence and survival of HSCs, but not HPCs, in response to regenerative stress. Thus, it is likely that under stress conditions, *Fhl2*-loss-induced decrease in quiescence and survival of HSCs under stress leads to the compromised self-renewal capacity of *Fhl2*-null HSCs. *FHL2* is one of the four genes that are most significantly induced in human peripheral blood lymphocytes after irradiation *ex vivo*^{54, 55}, indicating that *FHL2* is responsive to environmental stress. *FHL2* is likely to act as a critical regulator of hematopoietic homeostasis in response to various stresses. Previous

studies showed that *Fhl2* is dispensable for normal cardiac development but modifies responses to certain stress conditions in the adult heart⁵⁶. Collectively, these data suggest that *Fhl2* plays an important role in stress responses in multiple-tissues.

FHL2 is an important regulator of cell cycle and proliferation. *Fhl2* loss inhibits the proliferation of embryonic fibroblasts by down-regulating both positive and negative regulators of cell cycle³⁶. In addition, *Fhl2* regulates p21 (Cip1) expression in breast cancer cells³⁵, and it acts as a negative regulator of E4F1, a key player in control of cell proliferation in mammalian cells³⁴. Here, we document a role of *Fhl2* as an important regulator of the cell cycle of HSCs in response to stress. Of note, all three members of the CIP/KIP family including *p21 (Cip1)*, *p27(Kip1)* and *p57(Kip2)*, are down-regulated in *Fhl2*-null LSKs after transplantation. The CIP/KIP family functions as a negative regulator of G1-S progression by interacting with different cyclin-CDK complexes⁵⁷. *p21(Cip1)* was initially reported to play a role in regulating HSC quiescence⁴⁰ whereas recent studies showed that *p21(Cip1)* is critical for maintaining HSC quiescence under conditions of cellular stress rather than steady state^{58, 59}. *p27(Kip1)* deficiency alone has a limited effect on the regulation of HSC cell cycle⁴³. However, absence of *p27(Kip1)* accelerated the defective quiescence phenotype of *p57(Kip2)*-null HSCs^{44, 45}. Our results showed that forced expression of *p21 (Cip1)* or *p27 (kip1)* but not *p57 (kip2)* partially rescued the decreased quiescence of *Fhl2*-deficient LSKs. Therefore, it is likely that downregulation of *p21 (Cip1)* and *p27 (kip1)* at least partially mediates *Fhl2* deletion-induced quiescence loss in HSC-enriched population. Both *p27(Kip1)* and *p57(Kip2)* are direct targets of the TGF- β pathway⁶⁰, which can be regulated by *Fhl2*¹⁷. In addition, a previous study has shown that *FHL2* upregulates expression of p21 through the MAPK pathway in breast cancer cells³⁵. Additional studies are necessary to determine whether *FHL2* regulates CDK inhibitors through either or both of the MAPK and TGF- β pathways in HSCs.

In this study, we cloned a unique 5' flanking sequence of the *FHL2* transcript in hematopoietic cells. Analysis of the 5'-UTR regions of *FHL2*, *DRAL*, and *B-FHL2* revealed that the *FHL2* isoforms identified from different tissues each contain a unique 5' UTR region, indicating that the transcription of *FHL2* is under the control of different promoters, and is likely to be regulated by different transcriptional regulatory cis- and trans-elements in a variety of tissues. Collectively, transcriptional regulation of *FHL2* expression is dependent on cell context, which may be critical for the role of *FHL2* in "fine-tuning" a diversity of cellular processes. In addition, a CpG island was identified in the promoter region of *B-FHL2*. We demonstrated that hypermethylation of the promoter region of *FHL2* results in reduced expression of *FHL2* in the KG-1 myeloid leukemia cell line and primary AML cells, indicating that *FHL2* expression is regulated epigenetically. Our findings indicate that *FHL2* is down-regulated in a subgroup of AML and MDS patients. DNA hypermethylation is highly associated with both AML and MDS. It is likely that down-regulation of *FHL2* in a subgroup of MDS or AML patients also results from an aberrant DNA methylation.

In summary, our studies revealed that the expression level of *FHL2* is critical for maintaining HSC quiescence and survival under stress, and that deregulation of *FHL2* may predispose to the development of hematopoietic disorders, and may cooperate with mutations predisposing to leukemogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Institute of Health grants RO1 CA140979 (to Z.Q.) We thank the staffs in the UIC flow core facility for their assistance in cell sorting.

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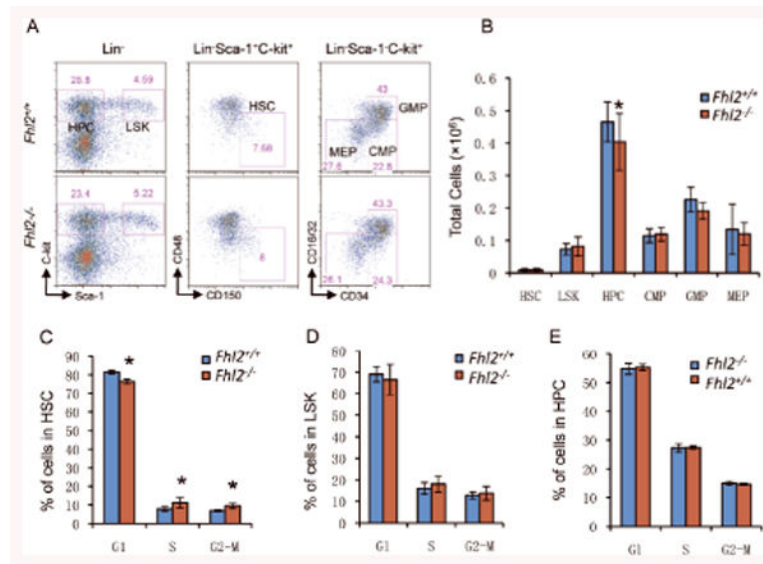


Figure 1. The effects of *Fhl2* loss on the number and cell cycle status of HSCs and HPCs in BM (A-B) The representative flow cytometric analysis of the frequency (A) and average absolute number (B) of HSCs, LSKs, HPCs, CMPs, GMPs and MEPs in BM cells from the control and *Fhl2*^{-/-} mice (n=5, *, P<0.05). (C-E) The histograms show the distribution of HSCs (C), LSKs (D) and HPCs (E) in G1, S and G2/M phase in BM from control and *Fhl2*^{-/-} mice (n=5, *, P<0.05). Cells were stained with DAPI. All mice were at age of 2 months. HPCs, LSKs, HSCs are defined as Lin⁻Sca-1⁻c-Kit⁺, Lin⁻Sca-1⁺c-Kit⁺ and LSK, CD150⁺CD48⁻ respectively. CMPs, GMPs and MEPs are defined as Lin⁻c-kit⁺Sca-1⁻ CD34^{+/lo}CD16/32^{int}, Lin⁻c-kit⁺Sca-1⁻ CD34⁺CD16/32⁺, and Lin⁻c-kit⁺Sca-1⁻ CD34⁻CD16/32⁻ respectively.

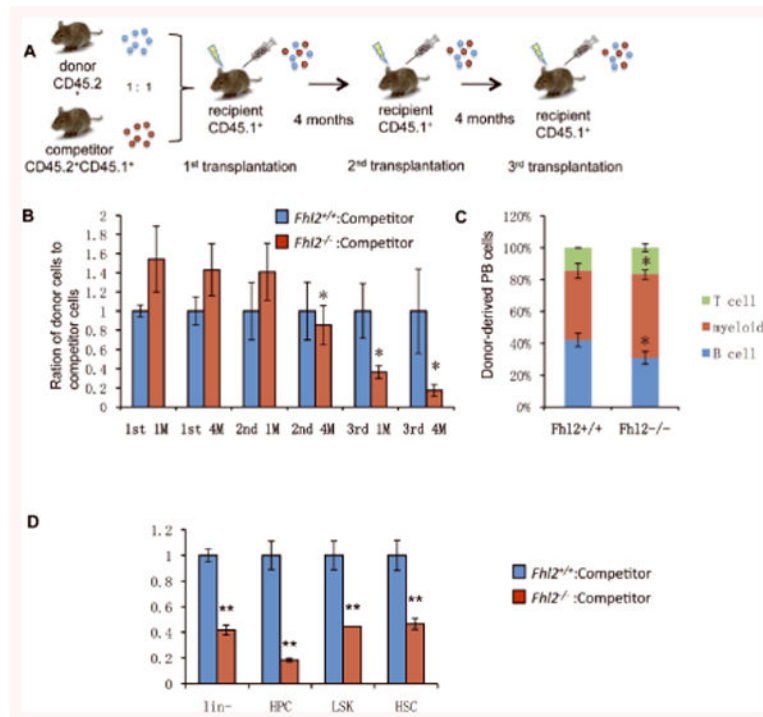


Figure 2. Loss of *Fhl2* leads to intrinsic functional defects and impaired long-term engraftment of HSCs

(A) The diagram for the experimental design. (B) Histogram showing the relative ratio of donor (CD45.2+) versus competitor PB cells (CD45.2+ /CD45.2+) at 1 to 4 months after 1st, 2nd and 3rd transplantation(*, P<0.05, n=5). (C) Lineage differentiation in the recipient mice 4 month after 3rd transplantation. (D) The relative ratio of donor (CD45.2+) versus competitor (CD45.2+ /CD45.2+) in Lin-, HPC, LSK, HSC in BM at 4 months after 3rd transplantation (n=5; *, P<0.05; **, P<0.01).

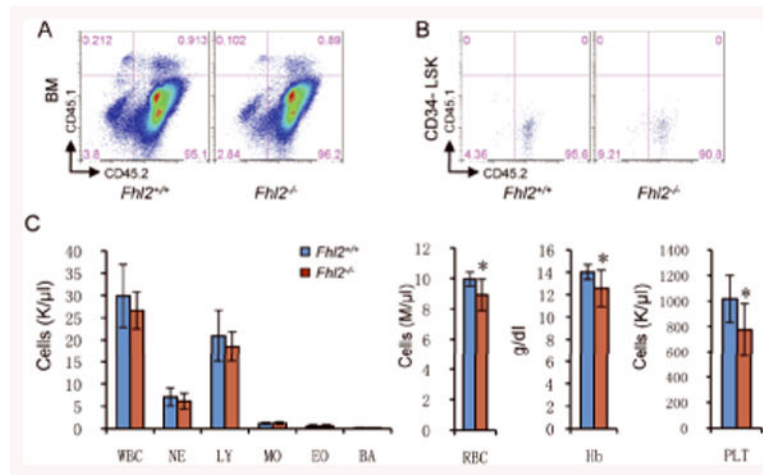


Figure 3. *Fhl2*-deficient chimeric mice developed abnormal hematopoiesis

(A-B) The representative flow cytometric analysis of engraftment of CD45.2⁺ *Fhl2*^{-/-} and control BM cells (A) and CD34⁺LSK cells (B) from CD45.1⁺ wildtype recipient mice (C) The number of white blood cells (WBC), neutrophils (NE), lymphocytes (LY), monocytes (MO), eosinophils (EO), basophils (BA), red blood cells (RBC), platelets (PLT), and hemoglobin (Hb) levels in PB from control and *Fhl2*^{-/-} chimeric mice (n=7; *, P<0.05).

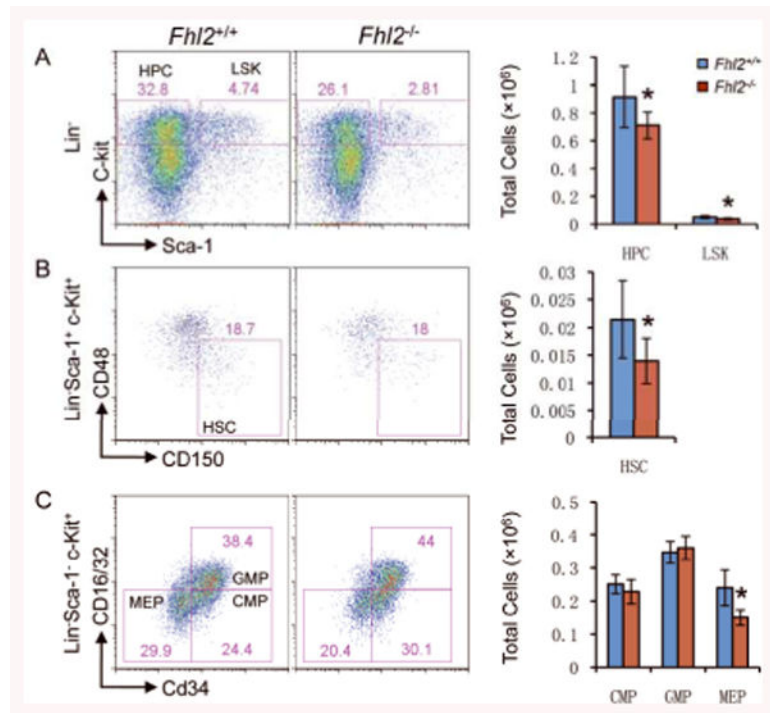


Figure 4. Decreased BM stem and progenitor cells in transplanted *Fhl2*^{-/-} mice
 (A-C) Representative FACS analyses (left) and average absolute number (right) of BM cells are shown for control *Fhl2*^{+/+} and mutant *Fhl2*^{-/-} mice. Numbers in the FACS plots indicate percentages in the gated population cells. All BM cells were analyzed from the chimeric mice at 7-8 months post-transplantation (n=5; *, P<0.05).

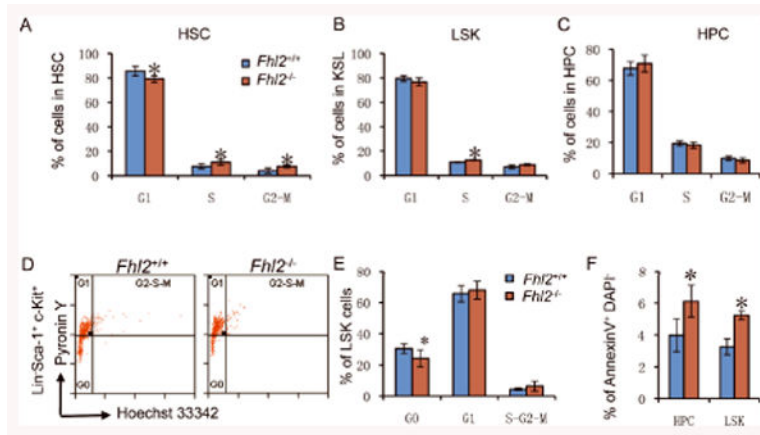


Figure 5. *Fhl2*-deficient HSCs had a reduced number of quiescent cells and displayed an increased frequency of proliferation and apoptosis after transplantation
 (A-C) The histograms show the distribution of HSCs (A), LSKs (B) and HPCs (C) in G1, S and G2/M phase in BM. Cells were stained with DAPI. (D) Representative flow cytometric analysis of LSK cells stained with Hoechst 33342 and Pyronin. (E) The histograms show the distribution of LSKs in G0, G1 and S-G2-M in BM. (F) The histograms depict the frequency of apoptosis in HPCs and LSKs in BM. All BM cells were isolated from the *Fhl2*^{+/+} and *Fhl2*^{-/-} chimeric mice (n=4-7; *, P<0.05) at 6-7 months post-transplantation.

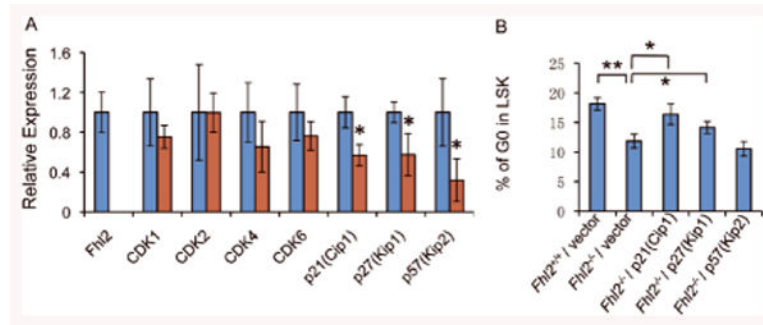


Figure 6. Down-regulation of CDK inhibitors in *Fhl2*-null LSKs

(A) qPCR analysis of the expression of CDKs and CDK inhibitors in *Fhl2*-null and wildtype control LSKs isolated from chimeric mice 6 months post-transplantation (n=3, p<0.05). LSKs are defined as Lin⁻Sca⁺c-Kit⁺ cells. (B) G₀ status of *Fhl2*-null and wildtype control LSKs with forced expression of *p21(Cip1)*, *p27(Kip1)* and *p57(Kip2)*. The G₀ status of infected cells was determined by flow cytometric analysis. **, P<0.005; *, P<0.05.

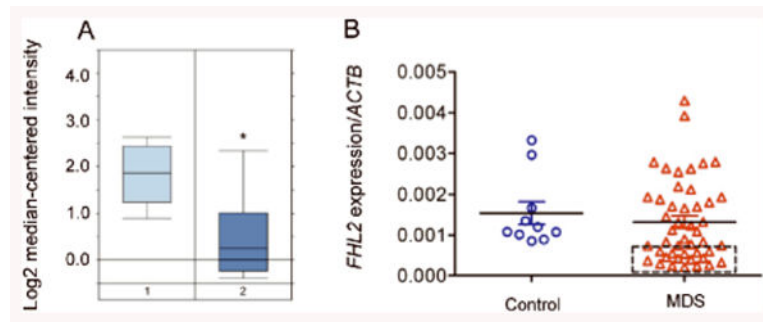


Figure 7. Downregulation of *FHL2* in patients with AML and MDS

(A) Expression profile of *FHL2* transcripts in PB mononuclear cells from 74 healthy individuals (left) and 542 AML patients (right) by microarray analysis ⁴⁶(Average fold change is -2.288. $P=6.28E-23$). (B) *FHL2* expression level in bone marrow cells from 46 patients with MDS and 10 healthy donors (Control, CTL) by qPCR. *ACTB* was used as the normalization gene. The square indicates the MDS patients who have a low *FHL2* expression level.

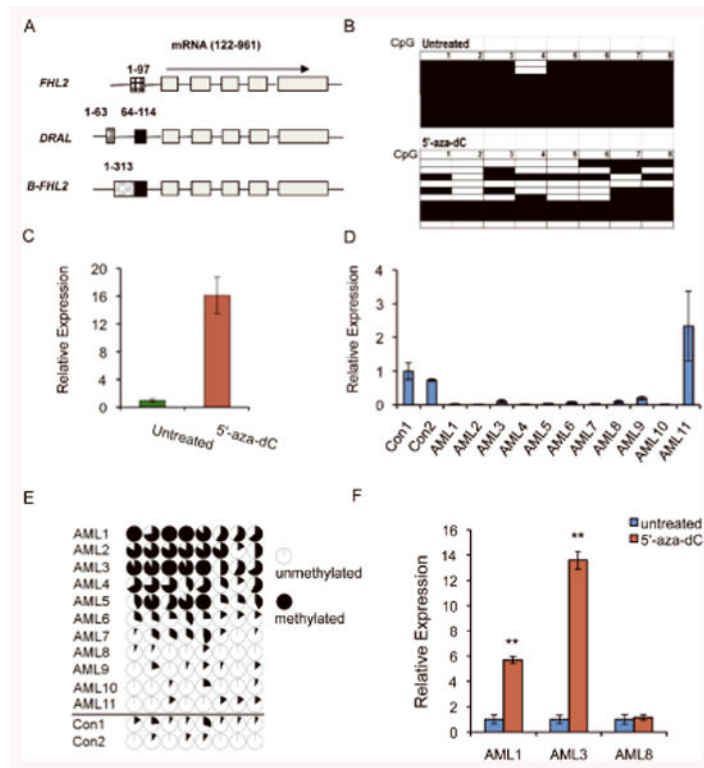


Figure 8. Expression of *FHL2* is associated with methylation status of the promoter region in myeloid leukemia cells

(A) *B-FHL2* has a unique promoter region. The boxes represent different exons, and the black line represents intronic sequences. The common exons encoding the *FHL2* protein, which are shared among these three transcripts, are shown in grey. The exons encoding the unique 5' UTR are shown with boxes with different patterns for each transcript. The black boxes within the *DRAL* and *FHL2* transcripts are identical. The numbers indicate the position within the GenBank nucleotide sequence [*FHL2* (gi:42403584), *DRAL* (gi:11761688), and *B-FHL2* (DQ307067)]. (B) Bisulfite sequencing of an amplicon from the promoter region of *B-FHL2* containing 8 CpG dinucleotides in untreated KG-1 cells or cells treated with 5'-aza-dC. Each row presents a clone. Filled squares indicate methylated CpGs, whereas open squares refer to unmethylated CpGs. (C) Relative expression of *B-FHL2* in untreated KG-1 cells, and cells treated with 5'-aza-dC was determined by qRT-PCR. The y-axis represents the ratio of fold-change in gene expression of *FHL2* between treated and untreated KG-1 cells. (D) The expression of *FHL2* in BM cells from AML patients and healthy individuals, as determined by qRT-PCR. (E) DNA methylation profile of the 8 CpG dinucleotides in the promoter region of *B-FHL2* gene in multiple AML samples (AML1-AML11) and 2 control samples (Con1, Con2) from healthy individuals. The cycles represent the average methylation level of a specific CpG dinucleotide. Unmethylated, partially methylated, and fully methylated are indicated by open cycles, partially blacked cycles and blacked cycles respectively. (F) Relative expression of *B-FHL2* in primary AML cells before and after treatment with 5'-aza-dC, as determined by qRT-PCR. The y-axis represents the ratio of *FHL2* level between treated and untreated cells.